

Effects of Sex Steroids on Dopamine Neurons in Cultured Hypothalamus and Preoptic Area Cells Derived from Neonatal Rats

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ABSTRACT—The effect of sex steroids on the preoptic area and hypothalamic dopamine neurons derived from neonatal rats within 12 hr after birth was studied in primary culture. Testosterone or estradiol-17 β was transiently added to the culture medium, and the cellular and medium dopamine contents were measured by an electrochemical detector connected with HPLC. Addition of testosterone (1 nM) for 3 days from day 2 of culture significantly increased cellular dopamine content and greatly increased medium dopamine concentration in the hypothalamic culture at day 14 of culture. Estradiol-17 β (1 nM) for 3 days from day 2 of culture also greatly increased medium dopamine concentration at day 14, but it did not alter cellular dopamine content in the hypothalamic culture. Neither testosterone nor estradiol-17 β altered the dopamine levels in the culture of the preoptic area cells. These results suggest that the property of cultured hypothalamic dopamine neurons was irreversibly altered by transient treatment with testosterone or estradiol-17 β , while that of cultured preoptic dopamine neurons was not affected.

INTRODUCTION

Morphological and functional sex differences are well documented in the central nervous system of mammals [16, 22]. Most of the sex differences in the brain are thought to be developed by steroid hormonal environment during the perinatal critical period in rats and mice, but not by genetic sex [17]. Androgen secreted from the testis during the critical period in male rats induces masculinization of the brain, while the brain without the androgenic influence develops into basic female-type. Exogenously administered testosterone or testosterone propionate during the critical period can shift the female-type brain to the male-type one. On the other hand, castration of male rats on the day of birth effectively generates the female-type brain instead of the male-type. It is considered that androgen not only directly acts on some discrete brain regions, but also manifests their indirect action through neural networks of androgen-sensitive neurons [9, 13]. Therefore, it is difficult by *in vivo* experiments to clarify whether the sex differences are really generated by direct action of androgens. The cell culture system is one of the effective methods for the study of direct action of androgen.

Dopaminergic systems are deeply concerned with the sexual differentiation of the brain. Perinatal administration of dopamine-related drugs demasculinizes male rats [11, 14]. The preoptic area (POA) and hypothalamus contain four dopamine (DA) neuron groups [4, 12]. Among such dopaminergic groups, the A12 group, also called tuberoinfundibular DA neurons, is located in the periventricular region of the

mediobasal hypothalamus including the arcuate nucleus. The neurons of this group extend their axons mainly to the median eminence, and inhibit the release of prolactin from the pituitary. Although no morphological sexual dimorphism has been found in this dopaminergic system, sex difference in the secretion and synthesis of DA has been reported [18]. The A14 group is located in the periventricular region between the POA and anterior hypothalamus. The exact role of the A14 group has not yet been established. DA neurons in this group appear to innervate both the luteinizing hormone-releasing hormone and GABA neurons in the medial POA [10] and the intermediate lobe of the pituitary [7]. Evident sexual dimorphism has been reported in the number and fiber density of DA neurons in the anteroventricular region of the A14 group by Simerly *et al.* [23].

The aim of the present study was to clarify the effects of sex steroids on DA neurons in the periventricular POA (including the A14) and hypothalamus (including the A12) in primary culture. We cultured the cells of each region derived from neonatal rats, and the cultured cells were transiently given testosterone or estradiol-17 β during early days of culture. Cellular and medium DA was measured at later days.

MATERIALS AND METHODS

Cell culture

Newborn rats of the Wistar/Tw strain (about 20 pups for one experiment) were used within 12 hr after birth. The sex of animals was disregarded, but the number of male and female rats was about the same. After decapitation, the brain was dissected out and pooled in the ice-cold serum-supplemented medium. The periventricular POA and hypothalamus were separately dissected out as indicated in Figure 1. Since A11 group is more caudally and A13 group is more dorsolaterally located in the hypothalamus, only small population of A11 and A13 DA neurons would be contaminated in

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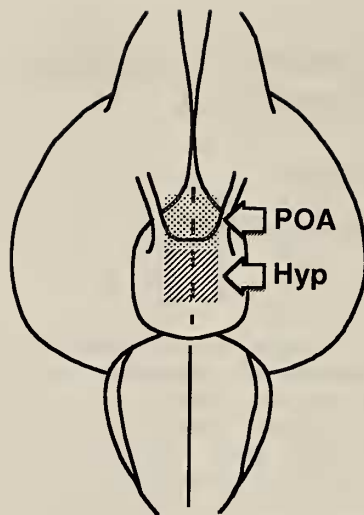


FIG. 1. Schematic diagram of dissected brain areas. Periventricular preoptic area (POA) was dissected out as a tissue clump of about 1 mm width, 1 mm height, and 1 mm depth cube at just rostral to the optic chiasma (hatched square). Periventricular hypothalamus (Hyp) was dissected out as a tissue clump of about 1 mm width, 1 mm height, and 1 mm depth cube at just caudal to the optic chiasma (dashed square).

dissected tissue pieces, if any. Pooled tissues of each brain region were dissociated by the method previously reported [26]. Briefly, the tissue clumps were digested in 1,500 PU/ml dispase (Godosyusei, Tokyo) in the serum-supplemented medium for 15 min at 37°C, followed by gentle pipettings. Enzymatic digestion and mechanical dissociation were repeated several times until most of the tissue clumps were dissociated. The dissociated cell suspension was kept in an ice bath during the dissociation step. After removing debris and remaining tissue masses by passing through a nylon mesh (40 μ m in pore size), cell suspension was centrifuged at 1,200 r.p.m. for 5 min. Collected cells were resuspended in a fresh culture medium, and cultivated in each well of 24-well multiwell culture plate (Falcon) at a density of 2.0×10^5 viable cells/well containing 0.4 ml of culture medium. The culture well was kept in an atmosphere of 5% CO₂-95% air at 37°C. Culture medium was replaced at 3-day intervals. To avoid possible effects of steroid hormones in the fetal calf serum (FCS), serum-free medium was routinely used except during the first 2 days of culture. Serum-supplemented medium was DME/F12 without phenol red (Sigma) supplemented with 100 IU/ml penicillin G, 100 μ g/ml streptomycin, additional 540 mg/ml glucose, and 15% fetal calf serum (FCS) in the serum-free medium. Serum-free medium was DME/F12 without phenol red supplemented with 100 μ M putrescine (Sigma), 30 nM sodium selenite (Sigma), 5 μ g/ml insulin (Sigma), 50 μ g/ml transferrin (Sigma), 100 IU/ml penicillin G, 100 μ g/ml streptomycin, and additional 540 mg/ml glucose. This serum-free medium was able to maintain the POA/hypothalamic neurons for a long period without any steroid hormone, if short preculture period with serum-supplemented medium was placed [25].

Following preculture in a serum-supplemented medium for 2 days, the medium was changed to the serum-free medium with or without steroid hormones. Testosterone (Sigma) or estradiol-17 β (Sigma) was dissolved in absolute ethanol, and diluted to 1.0 nM with the serum-free medium. The final concentration of the ethanol was 0.007%. The same percentage of ethanol was added to the control medium. Testosterone at the concentration of 1.0 nM has been

reported to facilitate the survival of neurons derived from neonatal rat POA [26]. After 3-day culture in a steroid hormone-containing medium, the medium was replaced to a serum-free medium without steroid hormones, and the cells were continued to be cultured. At day 14 of culture (9 days after the removal of steroids), cellular contents of DA and medium DA concentrations were measured. A few hypothalamic cultures were used for the observation of catecholamine neurons.

Staining of catecholamine neurons

Catecholamine neurons in culture were visualized by glyoxylic acid method [15]. At day 14 of culture, coverslips were briefly rinsed with PBS and immersed into 2% glyoxylic acid in PBS (pH 7.0) containing 20% sucrose for 20 min at 4°C in the dark. Then, the coverslip was dried by hot air for 5 min, and heated at 100°C for 5 min. The coverslip was mounted on a slide glass with liquid paraffin, and cells were observed under a fluorescent microscope. DA and noradrenaline are reported to emit blue fluorescence (emission max. = 475 nm) when excited by the light at the wavelength of 415 nm [15].

Measurement of dopamine (DA)

The content of DA was measured by an electrochemical detector connected with a high-performance liquid chromatography (HPLC). Reversed-phase column (TSK ODS-80Ts, Tosoh) was used for the analysis. The mobile phase was composed of 0.15 M NaH₂PO₄, 0.1 mM EDTA, 0.5% sodium octanesulphonic acid, and 5% methanol (pH 3.4). To concentrate and extract DA from the culture medium, method of Porter *et al.* [19] was used with a slight modification. The medium of each group was pooled, acidified with an equal volume of 0.1 M perchloric acid (PCA) - 0.4 mM sodium metabisulphate containing internal standard, 3,4-dihydroxybenzylamine (DHBA, Sigma), and stored at -80°C until measurement. After centrifugation at 9,000 \times g for 10 min and neutralization of supernatant with 1.0 M Tris buffer (pH 8.6), catecholamines were absorbed on activated alumina, back-extracted into 0.3 M PCA - 0.4 mM sodium metabisulphate, microfiltered (0.22 μ m in pore size), and 50 μ l aliquot of the extract was introduced into HPLC. To extract cellular DA content the cells in each well were harvested by a cell-scraper immediately after complete separation of the culture medium, and suspended with 200 μ l of 0.1 M PCA and 0.4 mM sodium metabisulphate containing DHBA. The suspended cells were sonicated at 4°C, and stored at -80°C until measurement. After centrifugation at 9,000 \times g for 10 min, the supernatant was microfiltered, and 50 μ l aliquot of this supernatant was directly introduced into HPLC. Recovery of DA (Sigma) in the present procedure was steadily about 85%. To calculate the concentration of DA, the method described by Felice *et al.* [6] was used. DA concentrations and contents were expressed as ng/ml and ng/well (mean \pm standard error), respectively. Student's t-test was used for analyzing cellular DA contents. For the measurement of DA in the medium, media of each group were all pooled, and therefore, no statistical analysis was applicable.

RESULTS

As we have previously reported [25], the number of neurons in the hypothalamus and POA culture at day 14 of culture was almost stable in the present study. When cultured cells were stained by glyoxylic acid [15] at day 14 of culture, DA neurons were observed as the cells that extended

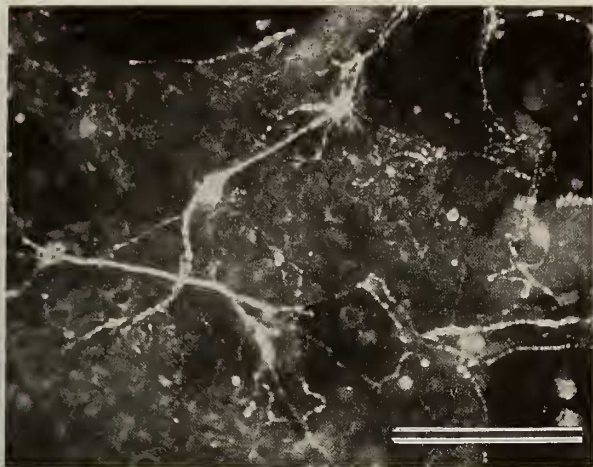


FIG. 2. Fluorescent microphotograph of catecholamine neurons in the hypothalamic cells at day 14 of culture stained by glyoxylic acid method. Catecholamine neurons show very long neurites with numerous branchings and varicosities. Bar=100 μ m.

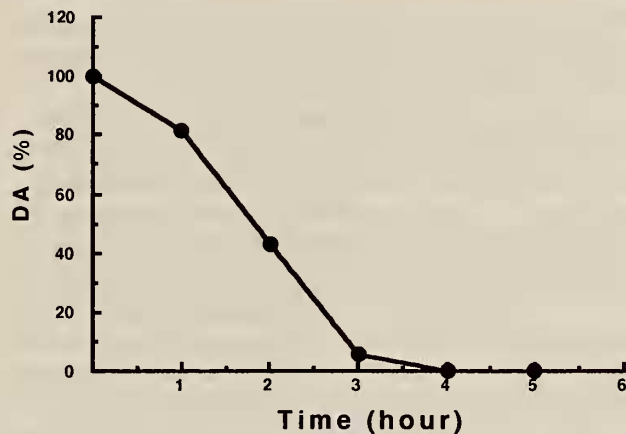


FIG. 4. Decrease of dopamine (DA) in the serum-free culture medium. Dopamine (5 ng/ml) was dissolved in the serum-free medium, and incubated at 37°C in the atmosphere of 5% CO₂-95% air. DA concentration is indicated as percentage of the initial value.

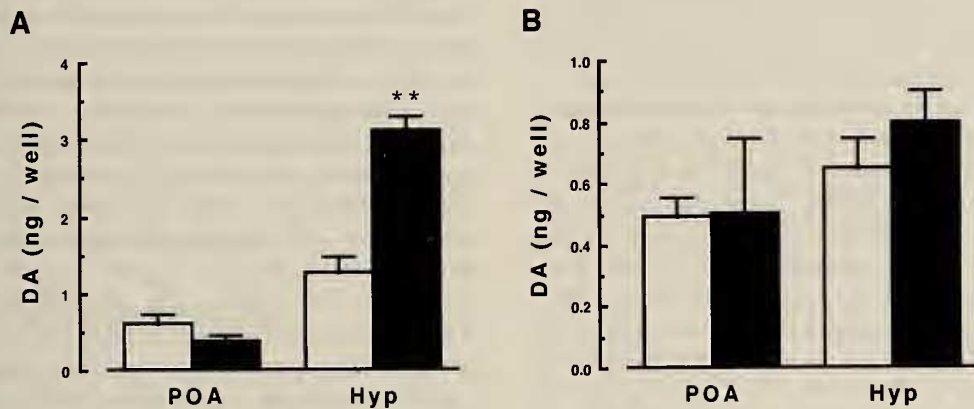


FIG. 3. Cellular dopamine (DA) contents after exposure to 1 nM testosterone (A) or estradiol-17 β (B) in cultured preoptic (POA) or hypothalamic (Hyp) cells at day 14 of culture. Each steroid was given for 3 days from day 2 of culture. Open columns indicate control cultures, and solid columns indicate steroid-treated cultures. Each column shows the mean \pm standard error of 3-4 samples. **P<0.01 (Student's t-test).

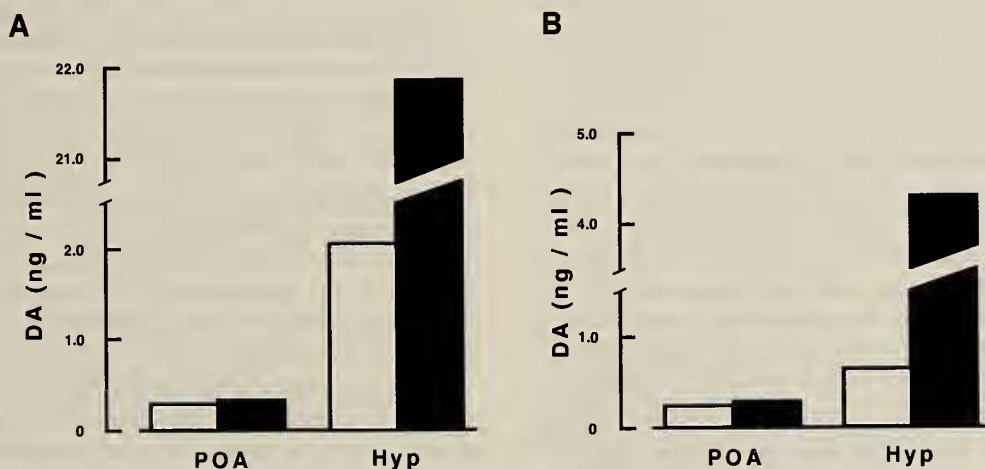


FIG. 5. Medium concentrations of dopamine (DA) after exposure to 1 nM testosterone (A) or estradiol-17 β (B) released from the preoptic (POA) or hypothalamic (Hyp) cells at day 14 of culture. Each steroid was given for 3 days from day 2 of culture. Medium was collected from 3-4 wells in each group, and content of DA was measured. Open columns indicate control cultures, and solid columns indicate steroid-treated cultures.

very long neurites with numerous varicosities and branchings (Fig. 2). However, no quantitative morphological comparison was examined in the present study.

As shown in Figures 3A and 5A, exposure to testosterone for 3 days from day 2 of culture increased both cellular contents and medium concentrations of DA in the hypothalamic culture. In the hypothalamic culture, the cellular DA contents were more than 2-fold greater in the cells exposed to testosterone than in the control cells at day 14 of culture (Fig. 3A). DA concentration in the medium was about 10-fold greater in the testosterone-exposed culture than in the control culture (Fig. 5A). Figure 4 shows the time-course decrease of standard DA in the serum-free medium at 37°C without any cell. DA rapidly decreased, and became undetectable after only 4 hrs of incubation. Therefore, measured DA concentrations in the media indicate the released DA during a short period before the termination of culture. Short-term exposure to estradiol-17 β tended to increase DA release from the hypothalamic culture, where DA concentration was about 7-fold greater in the medium of estradiol-exposed culture than the control culture (Fig. 5B). However, no significant difference was detected in the cellular DA content between estradiol-exposed and control cultures (Fig. 3B).

Exposure to testosterone failed to alter either cellular content or medium concentration of DA in the POA culture (Figs. 3A and 5A). Cellular DA content in testosterone-exposed POA culture was slightly lower than that in the control culture, but the difference was statistically not significant. The medium DA concentration of testosterone-exposed culture was almost the same as that of the control (Fig. 5A). Exposure to estradiol-17 β was not effective in altering cellular or medium DA contents (Figs. 3B and 5B).

DISCUSSION

In the present study, the cultured hypothalamic or POA cells derived from neonatal rats were transiently exposed to 1.0 nM testosterone or estradiol-17 β . Both cellular content and medium concentration of DA in the hypothalamic culture were increased after a long time lapse since the exposure to testosterone. Estradiol-17 β likewise elevated medium concentration of DA, but was not effective for cellular content. These results indicate that the hypothalamic DA neurons were irreversibly affected by the transient exposure to steroids in primary culture. Because estradiol-17 β also effectively elevated the medium DA concentration, conversion of testosterone to estradiol-17 β might be playing a role for the effect of testosterone in the alteration of the property of DA neurons. The effect of nonaromatizable androgen, 5 α -dihydrotestosterone, should further be tested for convincing this possibility. There are at least three possible explanations on the effect of sex steroids on the cultured hypothalamic DA neurons. The first is the effect on the survival of DA neurons. The second is the effect on the morphology of DA neurons. If axonal branching and/or varicosities of DA neurons are more numerous in the steroid-treated cultures,

more DA content in the cells and more release to the medium might occur. However, our preliminary observation (data not shown) by using glyoxylic acid method [15], which fluorochemically stained DA neurons, seemed to point out that the two possibilities were not feasible. The third possibility is the effect on the activity of each DA neuron. If the rate of synthesis and/or release of DA is increased, DA in the cells and culture medium should be increased. In the present study, cellular DA content was more than 2-fold greater in testosterone-treated cultures, while DA concentration in the culture medium was about 10-fold. This result fits well with the third possibility, suggesting that both synthesis and release of DA were increased, where the influence on the release of DA was more marked than on the synthesis.

Although morphological sex difference is absent, the activity of tuberoinfundibular DA neurons shows sexual dimorphism. Demarest *et al.* [5] reported that the concentration of DA in the median eminence was the same in male and female rats, but the basal rates of synthesis and turnover of DA in the median eminence were 2–3 fold greater in the female than in the male. DA content in the portal blood was several times higher in the female rat [3]. Similarly, while there is no sex difference in the number of DA neurons in the tuberoinfundibular system, the amount of tyrosine hydroxylase (TH) mRNA in the ovariectomized female rat was greater than in the male [1, 2]. Demarest *et al.* [5] stated that sex difference in the tuberoinfundibular DA system seems to be determined by the sex steroidal environment during neonatal period as are other sex differences in the brain. Sar [20] reported that considerable percentage of TH-immunopositive neurons in this region incorporated [³H]-estradiol-17 β , suggesting that the sex difference in this system was the consequences of the direct effect of sex steroids.

There are several reports on the effect of steroid hormones on the tuberoinfundibular DA neurons. Ovariectomy of female rats decreases the turnover rate of DA in the median eminence, and the administration of estradiol benzoate to the ovariectomized animal recovers the turnover rate [8]. Similarly, administration of estradiol [27] or testosterone [24] increases the turnover rate of DA in the median eminence in castrated male rats. Recently, Yamaguchi *et al.* [28] reported that short-term treatment with estradiol-17 β increased spontaneous release of [³H]-DA from cultured tuberoinfundibular cells. In our present experiments, testosterone and estradiol-17 β exerted a long-lasting stimulatory effect on the hypothalamic DA neurons. The effect of steroids on the hypothalamic DA neurons in the present study might reflect the sexual differentiation of the tuberoinfundibular DA neurons.

Apparent sexual dimorphism was reported in the number of DA neurons in the POA. The number of DA neurons in the anteroventral periventricular nucleus (AVPvN) is 3–4 fold more in the female rat than in the male [23]. This sexual dimorphism appears to be dependent on the perinatal levels of gonadal steroids, since orchidectomy of newborn males increased and treatment of newborn females

with testosterone decreased the number of TH mRNA-containing cells in the AVPVN [21]. However, there was no significant effect of transient sex steroid exposure in the present study on DA content in the periventricular POA cultures neither in the cells nor in the culture medium. Observation of Sar [20] that there was no coexpression between TH-immunoreactivity and [³H]-estradiol incorporation in the POA suggests the lack of direct response to estrogen in DA neurons in the POA. Gonadal steroid may indirectly influence on the sexual differentiation of DA neurons in the POA.

To conclude, transient exposure either to testosterone or estradiol-17 β induced long-lasting effects on the dopaminergic property of cultured hypothalamic cells derived from neonatal rats. Transiently administered testosterone for 3 days from day 2 of culture increased cellular DA content and medium DA concentration at day 14. Transiently administered estradiol-17 β also increased medium DA concentration, but cellular content was not affected. These data suggest that transiently administered sex steroids, testosterone or estradiol-17 β , irreversibly stimulated the basal level of DA synthesis and release in the hypothalamic culture of neonatal rats. Dopaminergic property of cultured cells derived from the periventricular POA was not affected by the treatments. Alterations of the tuberoinfundibular dopaminergic property by direct effects of sex steroids during critical period seem to be crucial for the determination of sex difference of DA neurons in this area.

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